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Short communication

In vitro liberation of carotenoids from spinach and Asia salads after different domestic kitchen procedures

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ABSTRACT

Green-leafy vegetables are rich in nutritionally important constituents including carotenoids. Their potential health benefits depend among others on their liberation from the plant matrix. The aim of the present study was to evaluate the effect of particle size and heat treatments on lutein and β-carotene liberation from spinach and Asia salads by applying an *in vitro* digestion protocol and UHPLC analysis. Reduction of particle size resulted in a three- to fourfold increase in liberation of lutein and β-carotene when comparing whole leaf and puree preparations of spinach. However, this positive effect was shown to be nullified by the severe heat impact during stir-frying of minced spinach, showing that domestic treatments need to be chosen carefully to maximise carotenoid liberation. Steaming significantly improved lutein liberation from Asia salads, but had no or a negative effect in spinach samples, possibly due to differences in liberation or degradation between the two plant matrices.

1. Introduction

Carotenoids are of importance as naturally occurring yellow to orange fat-soluble pigments (Maiani et al., 2009; Rao & Rao, 2007). As plant food components, they are associated with a decrease in the risk of chronic disorders such as cardiovascular diseases (Granado, Olmedilla, & Blanco, 2003) and specific types of cancer (Finley, 2005). Moreover, xanthophylls have a potential role in prevention and treatment of certain eye diseases such as age-related macular degeneration (AMD) and cataracts (Ma & Lin, 2010).

Dark green leafy vegetables are high in lutein and β-carotene and spinach consumption has been reported to increase both plasma lutein concentration and macular pigment optical density (Arnold, Jentsch, Dawczynski, & Bohm, 2013; Kopsell & Lefsrud, 2006). Moreover, a recently published meta-analysis revealed that an increase in lutein and zeaxanthin intake (from natural food sources) might be protective against late AMD (Ma et al., 2012). Carotenoid contents in these vegetables are highly affected by genetic and environmental factors such as species, cultivars, growing conditions and postharvest handling (Rodriguez-Amaya, 2015). Additionally, food preparation such as trimming clearly reduces carotenoid contents, whereas mashing or moderate heating often increases carotenoid availability, very likely due to enhanced extractability following maceration of cells (Bohn et al., 2015). However, severe heat treatments such as baking or sterilization might cause significant losses.

Liberation including disintegration of the plant matrix and cellular breakage are prerequisites for carotenoid bio-accessibility and thus availability for intestinal absorption. *In vitro* methods simulating gastro-intestinal digestion offer a simple and fast approach to screen both the liberation and accessibility potential. So far, results have been difficult to compare between research groups due to differences in published models and/or parameters (Alminger et al., 2014). Within the COST action INFOGEST, digestion experts therefore consolidated parameters for simulating digestion of food and published a harmonised digestion protocol (Minekus et al., 2014). Thus, the aim of the present study was to pre-screen in a first step carotenoid liberation from green-leafy vegetables by applying this standardised *in vitro* protocol. Spinach and Asia salads (also known as Japanese Greens) were subjected to the commonly used domestic heat treatments, steaming and stir-frying. Additionally, the effect of particle size on carotenoid release was evaluated on spinach, whereas Asia salads of different cultivars were compared. *In vitro* accessibility results obtained in a follow-up study will be reported elsewhere (Eriksen et al., submitted).

2. Materials and methods

2.1. Samples and sample preparation

A batch of fresh baby leaf spinach (*Spinacia oleracea*) was purchased from a local retailer (Migros, Switzerland). It was either used as whole leafs (cut with a knife in strips of 3 cm to mimic whole leaf consumption) or finely ground in a cutter

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(La Moulinette DPA 1, Solingen, Germany) to prepare a puree. Commercial minced spinach was bought from the same retailer, but was manufactured from another batch. Asia salads were grown at the Agroscope Research Station in Wädenswil, Switzerland. Seeds of the cultivars Green Boy and Tatsoi Green (both *Brassica rapa* ssp. *chinensis*), Mibuna Early (*B. rapa* ssp. *nipposinica*) as well as Bloody Mary (*Brassica juncea*) were cultivated in pots (8 pots/cultivar, 6 seeds/pot) in the greenhouse at $\geqslant 5$ °C under daylight in February/March 2013. Plants were harvested after 6 weeks when they reached a height of 15–20 cm. Damaged or yellow leaves were removed and all plants were well mixed and cut with a knife in strips of 3 cm. All preparations were divided into aliquots for further experiments.

For analytical determinations, one aliquot was directly flushed with liquid nitrogen (Messer Schweiz AG, Lenzburg, Switzerland), divided into three portions, ground to a fine powder at −20 °C in the cutter, and stored in amber plastic bottles at −20 °C until analysis. For the experiments on domestic heat treatments, two aliquots were immediately transferred into plastic bags and shock-frozen in liquid nitrogen (below referred to as raw); two aliquots were steamed for 3 min at 100 °C, transferred into plastic bags and shock frozen (steamed); two aliquots were stir-fried with peanut oil (2.5 g/75 g vegetable) for 6 min under constant stirring and shock frozen (stir-fried). Whole leaves and spinach puree were processed in triplicates. All samples were stored at −20 °C in the dark until *in vitro* digestion experiments.

2.2. In vitro digestion

To evaluate carotenoid liberation, the *in vitro* digestion procedure based on the standardised COST Infogest protocol (Minekus et al., 2014) with electrolyte concentrations adapted from Kopf-Bolanz et al. (2012) was applied. Briefly, 5 g of sample was weighed into an amber screw-capped glass tube and incubated at pH 7.0 ± 0.2 for 10 min with 5 mL of simulated saliva fluid containing 1 mg of human saliva α-amylase (A1031, Sigma–Aldrich, Buchs, Switzerland). Next, 10 mL of simulated gastric fluid containing 3.9 mg of porcine gastric pepsin (P7012, Sigma–Aldrich) were added, pH was adjusted to 3.0 ± 0.2, the headspace flushed with nitrogen and the sample incubated for 2 h. Finally, after addition of 20 mL of simulated intestinal fluid containing 220 mg of porcine pancreatin and 334 mg of bile from bovine and ovine sources (P7545 and B8381, both Sigma–Aldrich), pH adjustment to 7.0 ± 0.2, and nitrogen flushing, the samples were incubated for another 2 h. All incubations were carried out at 37 °C in a shaking water bath at 90 strokes/min. Gastric and intestinal incubations as well as all following steps were done under red light. After incubation, the samples were immediately centrifuged twice for 10 min (4495 g, Haereus Multifuge 3RS+, Thermo Fischer Scientific, Reinach, Switzerland) to remove undigested solids and oil droplets, which are not considered available for absorption. 5 mL of the aqueous fraction including micelles were freeze-dried in the dark and immediately afterwards re-dissolved in extraction medium for carotenoid quantification. Each aliquot of domestic heat preparation was incubated twice.

2.3. Carotenoid quantification

The extraction procedure was performed under red light. Lutein and β-carotene contents were determined after extraction with methanol/acetone (1:1, v/v) containing 0.01% BHT as described previously (Reif, Arrigoni, Schärer, Nyström, & Hurrell, 2013). Starting materials were homogenised for 30 s in a Polytron blender at full speed under constant nitrogen supply followed by 30 min ultrasonic treatment, as were re-dissolved digestion supernatants. Aliquots of the extracts were filtered (Nylon syringe filters, pore size 0.22 μm) into brown UPLC-vials and immediately analysed.

UHPLC-PDA analysis was carried out based on the method described by Chauveau-Duriot, Doreau, Nozière, and Graulet (2010), modified by Eriksen et al. (submitted), by using an Acquity system (UPLC™, Waters Corporation, Milford, USA). In brief, separation was achieved on an Acquity UPLC column (HSS C18, 1.8 μm, 2.1 × 150 mm with a Vanguard pre-column HSS C18, 1.8 μm, 2.1 × 50 mm, Waters) with an ammonium acetate-acetonitrile-dichloromethane-methanol gradient. Quantification was based on external standards purchased from Carotenature (Lupsingen, Switzerland).

2.4. Data analysis

Carotenoid content in starting material is presented as mg carotenoid/100 g fresh material. *In vitro* carotenoid liberation was calculated as the fraction (%) of carotenoid released from the matrix to the total carotenoid content in the respective starting material. Data are presented as mean ± SD. Statistical difference for pairwise comparisons was calculated by student's *t* test, whereas for triple comparisons ANOVA followed by the Tukey–Kramer test was applied. *P* values <0.05 were considered significant.

3. Results and discussion

3.1. Carotenoid content

Table 1 reports the contents of lutein and β-carotene in the edible part of spinach and Asia salads. As for raw vegetables, carotenoid concentrations varied considerably between the two batches of spinach (as described in Section 2.1). This might be due to differences in cultivar, season, growing location, cultivation practise or industrial processing (Maiani et al., 2009). However, maturity is likely to play a key role, since minced spinach contained approx. 40% less lutein and β-carotene than whole leaves and puree which were produced from baby leaf spinach. This is in agreement with an earlier published investigation on New Zealand spinach showing a similar decrease from young to mature leaves (de Azevedo-Meleiro & Rodriguez-Amaya, 2005) and can be explained by changes in the leaf:rib (stem) ratio. Stems and ribs, which constitute a lower proportion in baby leaves, have been shown to be basically carotenoid-free (Reif, Arrigoni, Schärer et al., 2013). Similarly, stems of Tatsoi Green and Bloody Mary were more distinct than those of Mibuna Early and Green Boy, thus resulting in lower lutein and β-carotene contents. Taking differences in cultivars and agronomical practices into account, carotenoid contents of raw green leaves presented here, can be considered in good agreement with earlier published spinach (Kopsell & Lefsrud, 2006; Reif et al., 2012) and Asia salad data (Krumbein, Schonhof, & Schreiner, 2005; Reif, Arrigoni, Berger, Baumgartner, & Nyström, 2013).

Steaming affected the carotenoid content in the investigated vegetables differently (Table 1). No effect was seen for Asia salads and spinach puree. A rather small, but significant decrease due to steaming was observed for whole leaves, while the contents of both carotenoids in minced spinach were increased by more than 50%. Contradictory results have also been reported in the literature. Bunea et al. found no significant effects of boiling and steaming of spinach (Bunea et al., 2008), whereas Mazzeo et al. (2011) stated a significant increase in β-carotene due to steaming. Interestingly, frozen spinach had been used as starting material in the latter study as it was the case for our minced spinach. Whether industrial preparation (i.e. mincing, blanching and frozen storage) had an impact on carotenoid extractability after additional steaming in these samples, remains speculative. Interesting findings were obtained after stir-frying. Both lutein and β-carotene contents

Table 1
Carotenoid content (mg/100 g edible matter) of differently processed green leafy vegetables.

	Lutein			β-Carotene		
	Raw Mean ± SD	Steamed Mean ± SD	Stir-fried Mean ± SD	Raw Mean ± SD	Steamed Mean ± SD	Stir-fried Mean ± SD
Spinach						
Whole leaves	11.08 ± 0.11[b]	9.65 ± 0.15[a]		6.00 ± 0.11[b]	5.16 ± 0.11[a]	
Minced[#]	6.30 ± 1.02[a]	10.77 ± 1.06[b]	13.84 ± 0.17[*,c]	3.47 ± 0.62[a]	5.29 ± 0.28[b]	7.65 ± 0.23[*,c]
Puree	10.42 ± 0.26[a]	10.82 ± 0.10[a]		5.83 ± 0.15[a]	5.63 ± 0.35[a]	
Asia salads						
Green Boy	6.72 ± 0.04 [a]	6.89 ± 0.18[a]	10.23 ± 0.24[b]	3.71 ± 0.16[a]	3.80 ± 0.20[a]	5.44 ± 0.23[b]
Tatsoi Green	4.81 ± 0.35[a]	5.25 ± 0.34[a]	8.73 ± 0.24[b]	2.49 ± 0.20[a]	2.77 ± 0.16[a]	4.25 ± 0.35[b]
Mibuna Early	5.53 ± 0.65[a]	5.49 ± 0.16[a]	7.28 ± 0.31[b]	3.27 ± 0.34[a]	3.20 ± 0.08[a]	4.17 ± 0.39[b]
Bloody Mary	4.98 ± 0.18[a,b]	4.74 ± 0.43[a]	5.89 ± 0.57[b]	2.50 ± 0.13[a]	2.72 ± 0.17[a]	3.39 ± 0.36[b]

Data represent mean values ± SD of triplicates except:
[*] $n = 2$. Significant differences ($P < 0.05$) of means between treatments are indicated with different letters for each carotenoid.
[#] Minced spinach was prepared from a different batch than whole leaves and puree.

were found to be more than doubled in stir-fried minced spinach and between 20% and 70% higher in Asia salads respectively, when compared to raw leaves. de Sá and Amaya-Rodriguez reported concentrations of these two carotenoids to be up to 20% and around 30% higher when stir-frying endive and kale respectively (de Sá & Rodriguez-Amaya, 2004). This increase is partly attributed to the water evaporation of 15–24% (results not shown) while during steaming the water content remained constant. Whether stir-frying facilitated carotenoid extractability (de Sá & Rodriguez-Amaya, 2004), remains speculative.

3.2. Carotenoid liberation by in vitro digestion

Due to their hydrophobic nature, the absorption of carotenoids is generally rather low and this may be partially due to low liberation from the food matrix. When this liberation was simulated by *in vitro* digestion, lutein was more easily released from the food matrix than β-carotene (Figs. 1 and 2). This is in agreement with earlier reports and is likely due to the higher hydrophilicity of xanthophylls compared to carotenes (Garrett, Failla, & Sarama, 1999).

Fig. 1 shows the effects of particle size and heat treatment on carotenoid liberation of spinach. Lutein liberation significantly increased with decreasing particle size for both raw and steamed samples ($P < 0.05$), whereas a significant increase in β-carotene liberation was only seen for puree. Steaming negatively affected lutein liberation from whole leafs and puree produced from the same starting material (as described in Section 2.1), while for minced spinach no difference was observed. Effects on β-carotene liberation after digestion are generally smaller, showing only a significant decrease due to steaming for whole leaves. Liberation data reported in literature refer to homogenised spinach only, although particles in the stomach are likely to be larger (Garrett, Failla, & Sarama, 2000), in particular when leafy vegetables are ingested. Similar observations have been reported for the lutein content in digesta of boiled or microwaved spinach (O'Sullivan, Ryan, Aherne, & O'Brien, 2008). Lutein transfer to the aqueous fraction reported for a cooked vegetable meal containing finely chopped spinach as the sole lutein source (Garrett et al., 2000) and a similarly composed baby meal (Garrett et al., 1999) are in good agreement with our data, whereas approximately twice as much β-carotene was liberated from these meals. This can possibly be explained by the fact that both meals contained some fat which is known to improve incorporation of β-carotene but not lutein into micelles (Nagao, Kotake-Nara, & Hase, 2013). Recoveries in digesta of homogenised, microwaved spinach (rather reflecting stability than liberation) have been reported to be around 80 and 70% for lutein and β-carotene respectively (Chitchumroonchokchai, Schwartz, & Failla, 2004). In contrast, only

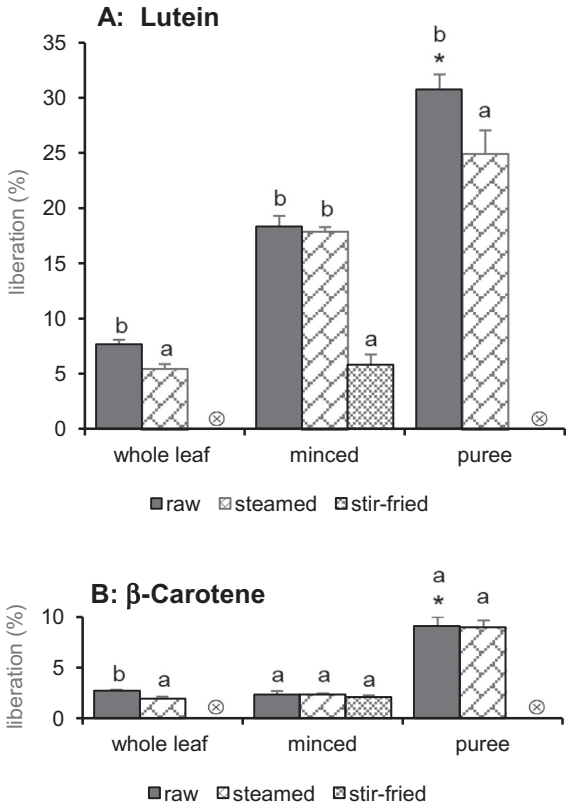

Fig. 1. Effect of domestic heat treatment and particle size on lutein (A) and β-carotene (B) liberation from spinach after *in vitro* digestion simulating upper gastro-intestinal enzymatic degradation. $n = 3$ for whole leaf and puree of baby leaf spinach (except *: $n = 2$); $n = 4$ for minced spinach. Significant differences ($P < 0.05$) of means between treatments are indicated with different letters. ⊗ : not investigated. Minced spinach was prepared from a different batch than whole leaves and puree.

about 5% of lutein, but 25% of β-carotene were found to be liberated in a similarly processed spinach sample (Granado-Lorencio et al., 2007), whereby liberation might have been affected by ongoing enzymatic and chemical reactions since carotenoids were quantified after overnight decantation of the supernatant at room temperature. This clearly shows that standardising digestion parameters are crucial to allow data comparison (Biehler & Bohn, 2010).

A different pattern regarding the effect of steaming was seen for Asia salads (Fig. 2). Lutein liberation was found to be significantly higher from steamed than from raw salads (except Mibuna Early which only showed a trend). Whether this is a species (*Brassica*

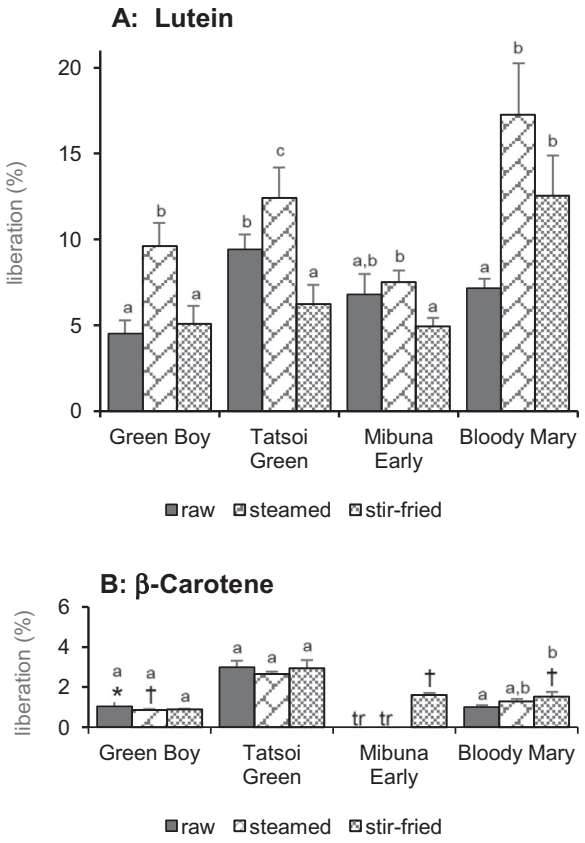

Fig. 2. Effect of domestic heat treatment and cultivar on lutein (A) and β-carotene (B) liberation from Asia salads after *in vitro* digestion simulating upper gastrointestinal enzymatic degradation. $n = 4$ (except *: $n = 2$; †: $n = 3$). Significant differences ($P < 0.05$) of means between treatments are indicated with different letters. tr: traces.

vs. *Spinacia*) related matrix effect due to differences in susceptibility to carotenoid release and/or degradation after moderate heating remains to be elucidated. As for spinach, β-carotene liberation from Asia salads was not improved by steaming and remained on a very low level.

Stir-frying did not affect carotenoid liberation from Green Boy and Mibuna Early, but significantly increased it from Bloody Mary when compared to liberation from raw leaves (Fig. 2). Since this domestic treatment led to a significant increase in lutein as well as β-carotene content (Table 1), the absolute amount of carotenoids liberated per 100 g edible matter after stir-frying is considerably higher than in the raw vegetable (data not presented). This can be considered positive from a nutritional point of view. It has to be mentioned, however, that this observation refers to samples mimicking whole leaf consumption (as described in Section 2.1). In contrast, lutein liberation from stir-fried minced spinach was significantly reduced by a factor 3 compared to raw or steamed spinach (Fig. 1). Obviously, the positive effect of particle size reduction on carotenoid liberation was overbalanced by their loss due to oxidation and isomerisation caused by the severe heat treatment (Bohn et al., 2015). Thus, domestic treatments need to be selected carefully to maximise carotenoid liberation.

Although belonging to the same botanical family and having been treated identically, some differences in lutein liberation patterns of Asia salads can be observed (Fig. 2). Both domestic heat treatments led to a considerably enhanced lutein liberation in leaves of *B. juncea* (Bloody Mary), whereas changes in the three *B. rapa* salads were much less pronounced. More experiments would be necessary to elucidate whether this observation can be explained by a species or a cultivar effect.

4. Conclusions and outlook

Carotenoid liberation from digested green-leafy vegetables is generally low, but can be considerably enhanced by particle size reduction. Mild heat treatments offer another possibility to facilitate liberation from the matrix, which could possibly be further improved by the addition of a lipid source. Further experiments are necessary to establish optimal domestic processing combinations to produce realistic and palatable vegetable dishes with a high carotenoid bioavailability potential.

The presented results support the usefulness of *in vitro* digestion as a simple tool for screening carotenoid liberation from differently processed green-leafy vegetables. However, quantification of carotenoids in the micellar fraction obtained by filtering the supernatant allows to assess *in vitro* accessibility thus offering a potentially more physiologically relevant prediction of the bioavailability potential (Eriksen et al., submitted).

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